Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 7575–7579, December 1982
Neurobiology

Use of a monoclonal antibody to purify the tetrodotoxin binding component from the electroplax of *Electrophorus electricus*

(action potential/neurotoxin binding/protein A/sodium channel)

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Communicated by Ray D. Owen, August 5, 1982

ABSTRACT **The tetrodotoxin binding component of the voltage-sensitive sodium channel from *Electrophorus electricus* electroplax was purified by using a monoclonal antibody. An impure preparation of tetrodotoxin binding component was mixed with the pure monoclonal antibody, and the immune complex so formed was isolated by affinity chromatography on a protein A-Sepharose column. Excess antibody was removed by ion-exchange chromatography. The purified material has a specific activity of over 1,800 pmol of [^{3}H]tetrodotoxin bound per mg of protein. By assuming that the immune complex has a stoichiometry of 1:1, this specific activity then represents an actual specific activity of 3,000 pmol of [^{3}H]tetrodotoxin bound per mg of eel electroplax protein, or 75% of the theoretical specific activity expected for a pure toxin binding component of M_r 250,000. The peptide composition of the purified material was simple with the predominant species present being of $M_r \approx 250{,}000$. Minor components were also present with M_rs of ≈95,000, ≈44,000, and ≈23,000.**

The action potential of excitable tissue results from transient changes in the permeability of the membranes to Na^+ and K^+ ions (1). The increase in Na^+ conductance is mediated through a voltage-regulated Na^+ channel (reviewed in refs. 2 and 3), which can be studied using the specific neurotoxins tetrodotoxin (TTX) and saxitoxin. These toxins bind reversibly and with high affinity to part of the Na^+ channel involved in Na^+ permeation; they block ion conductance but do not perturb the structures involved in channel gating.

The TTX binding component of this channel has been solubilized and partially purified from the electric organ of *Electrophorus electricus* (4, 5), from the sarcolemma of rat skeletal muscle (6), and from mammalian brain (7, 8). Agnew *et al.* suggested that a polypeptide of M_r 250,000 constituted part of the Na^+ channel, for TTX binding activity was correlated in a number of fractionation procedures with the appearance of this component on $NaDodSO_4$/polyacrylamide gels (5). Hartshorne and Catterall, using lectin affinity chromatography, obtained a partial purification of material from rat brain and identified protein components of M_rs 270,000 and 38,000 (7). Barchi *et al.*, using a similar approach, showed that in rat muscle peptides of M_r 64,000, 60,000, and 54,000 are components of a sarcolemma Na^+ channel preparation (6), although the same authors in a recent report (9) have stated that their preparation contains a large glycoprotein that runs anomalously as a diffuse band at positions between M_rs 140,000 and 220,000, a M_r 45,000 component, and a doublet at M_r 37,000 (data not shown).

Additional insight into Na^+ channel peptide composition has come from studies with polypeptide toxins that bind to a voltage-sensitive site on the channel. Beneski and Catterall, using a photoactive derivative of scorpion toxin, labeled components of M_rs ≈ 250,000 and 32,000 in rat brain (10), although a single component of M_r 250,000 was labeled in neuroblastoma cells.

However, the purification procedures used to date have only produced preparations that are 30–50% pure, as measured by TTX or saxitoxin binding (4, 6, 7). This is of insufficient purity for structural studies. In addition, it is difficult to identify unequivocally those polypeptides that comprise the TTX binding component. Recently, a monoclonal hybridoma cell line secreting antibody against the saxitoxin binding component of *E. electricus* electric organ was described (11). The antibody immunoprecipitated the toxin binding activity and apparently specifically bound to a component of M_r 250,000 on $NaDodSO_4$/polyacrylamide gels. In this report, we describe the use of this antibody to purify the toxin binding component from *E. electricus*.

MATERIALS AND METHODS

Electric organs from small or medium sized *E. electricus* (World Wide Scientific Animals, Ardsley, NY) were frozen in liquid nitrogen immediately after dissection and stored at −80°C until use. [^{3}H]TTX (specific activities of 165 and 68 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) and deionized Lubrol-PX (Sigma) were prepared as described (4). L-α-Phosphatidylcholine (type V-F from egg yolk) and chromatography media were from Sigma. Purified *Staphylococcus aureus* protein A and protein A-Sepharose 4B-CL were obtained from Pharmacia. All other materials were obtained as described (4).

IgG from the hybridoma clone VD10 (11) was purified from ascites tumor fluid by a slight modification of the procedure of Ey *et al.* (12): 0.1 M sodium phosphate (pH 8) containing 0.05% NaN_3 was added to an equal volume of ascites fluid, which was then circulated through a protein A-Sepharose 4B-CL column for at least 2 hr; the column was then extensively washed with the phosphate buffer, and pure antibody was eluted from the column with 0.1 M sodium citrate (pH 5.0) containing 0.05% NaN_3. The fractions containing protein were pooled, the pH was adjusted to 7.5 with 1 M NaOH, and the mixture was dialyzed against 1 liter of 0.1 M sodium phosphate (pH 7.5) containing 0.05% NaN_3 for 3 hr with three changes. After dialysis, the IgG was concentrated to 1.5 ml by membrane ultrafiltration (Amicon XM-50, 15–20 psi; 1 psi = 6,895 Pa).

A partially purified preparation of TTX binding component, obtained by ion-exchange chromatography, was used as the starting material for incubation with VD10 IgG (11) and subsequent purification by protein A-Sepharose chromatography. This was prepared by a modification of the procedure of Agnew

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Abbreviations: TTX, tetrodotoxin; Lubrol/PtdCho, Lubrol/phosphatidylcholine (7:1 molar ratio).

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Table 1. Purification of the TTX-binding component–immune complex

	[^{3}H]TTX bound, pmol	Protein, mg	Specific activity*	% initial binding activity
1% Lubrol extract	7,179	324	22	100
Preparation I	3,861	7.8	495	54
Preparation I (concentrated by ultrafiltration)	1,872	2.9	646	26
Protein A-Sepharose				
Total	1,604			22
pH 7.5 elution	1,069	—	—	
pH 6 elution	535	—	—	7.5
DEAE-Sephadex (1 ml)				
Total	220			3.1
Fraction† 17	96	0.053	1,811 (2,898)‡	
18	48	0.032	1,500 (2,400)‡	
19	30	0.016	1,875 (3,000)‡	
20	20	0.011	1,818 (2,909)‡	

* Expressed as pmol of [^{3}H]TTX bound per mg of protein.
† Fractions from 0.4 M NaCl elution.
‡ Calculated by assuming a 1:1 complex of TTX binding component (M_r, 250,000) to IgG (M_r, 150,000).

et al. (4): packed eel electroplax membranes were obtained as described (5) except that 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM iodoacetamide were added. A 1% Lubrol-PX extract of these membranes was treated with DEAE-Sephadex as described (4), except that the buffer used was 50 mM potassium phosphate (pH 7.5) containing 0.03% Lubrol/phosphatidylcholine (Lubrol/PtdCho, 7:1 molar ratio) and 0.2 M KCl. In all stages of this purification, the following protease inhibitors were added: 0.5 mM iodoacetamide, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1 μM pepstatin A. After washing with buffer, the DEAE-Sephadex was poured into a column, and the [^{3}H]TTX binding activity was eluted with buffer containing 0.5 M KCl. The fractions containing [^{3}H]TTX binding activity were pooled and dialyzed against 1 liter of 0.1 M sodium phosphate (pH 7.5) containing 0.03% Lubrol/PtdCho, 0.05% NaN_3, and protease inhibitors for 3 hr with three changes. After dialysis, 50 nM [^{3}H]TTX was added, and the material was concentrated to 1.5 ml by membrane ultrafiltration (Amicon XM-300, 10 psi). This material will be referred to as preparation I.

The concentrated VD10 IgG was added to preparation I and [^{3}H]TTX was added to give a final concentration of 1 μM. After 8–12 hr of incubation at 4°C, the material was further fractionated by affinity chromatography with protein A-Sepharose and DEAE-Sephadex chromatography as described in *Results*.

For comparison a semipurified preparation of TTX binding

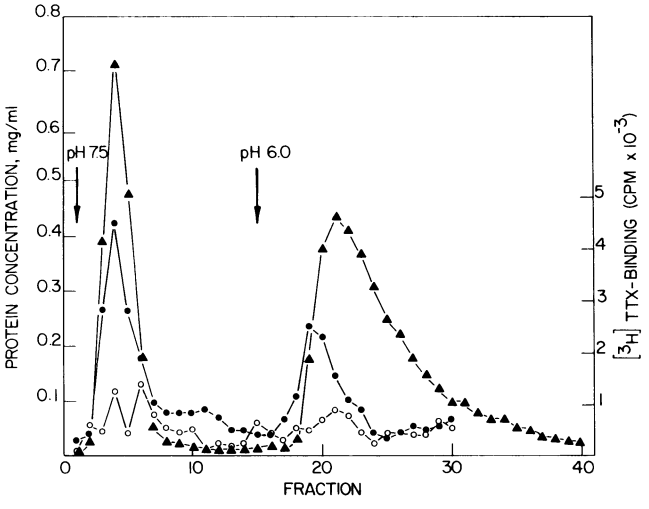

FIG. 1. Protein A-Sepharose 4B-CL chromatography of the TTX binding component–VD10 IgG immune complex. ●, [^{3}H]TTX binding; ○, [^{3}H]TTX binding in the presence of 2 μM unlabeled TTX; ▲, protein concentration determined by the fluorescamine assay (13). Preparation I was mixed with purified VD10 IgG (4.5-ml final volume contained 0.43 μM [^{3}H]TTX binding sites and 11 μM IgG). After 8 hr at 4°C, the mixture was applied to a 9-ml protein A-Sepharose 4B-CL column and eluted with 0.1 M sodium phosphate (pH 7.5) containing 0.1% Lubrol/PtdCho, 0.05% NaN_3, 25 nM [^{3}H]TTX, and protease inhibitors. The column was pumped at 7 ml/hr for fractions 1–3 and then at 30 ml/hr subsequently. After 15 fractions had been collected (2.5 ml each), the elution buffer (same composition as above) was changed to pH 6. A further 25 fractions were collected. [^{3}H]TTX binding activity was not affected by decreasing the pH from 7.5 to 6.

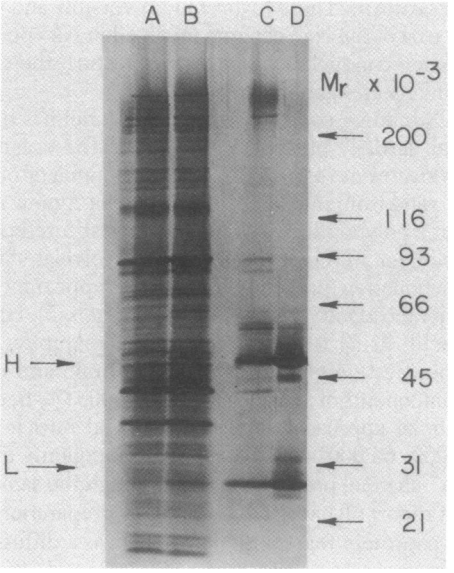

FIG. 2. Gradient $NaDodSO_4$/polyacrylamide gel (4–15%) electrophoresis of protein A-Sepharose column fractions under reducing conditions. Lanes: A, preparation I before incubation with IgG; B, peak fraction (no. 4) of the pH 7.5 elution in Fig. 1; C, peak fraction (no. 19) of the pH 6 elution in Fig. 1; D, fraction 22 of the pH 6 elution in Fig. 1. Samples were boiled in 62.5 mM Tris, pH 6.8/3% $NaDodSO_4$/10% glycerol immediately after purification and stored at −80°C until gel electrophoresis. The gel was stained with silver by the method of Oakley *et al.* (15). The molecular weights of standard proteins and the heavy (H) and light (L) chains of IgG are indicated.

component was prepared by conventional methods exactly as published (4) with a batch ion-exchange chromatography procedure followed by Sepharose 6B chromatography. The specific activities of the peak fractions after rechromatography on a Sepharose 6B column ranged from 1,730–2,150 pmol of [^{3}H]-TTX bound per mg of protein. This preparation will be referred to as preparation II.

Protein concentration was determined by the fluorescamine method (13) with bovine serum albumin as standard. [^{3}H]TTX binding was measured by the rapid gel filtration method as described (4). Electrophoresis was performed under reducing conditions (50 mM dithiothreitol) with a linear gradient (4–15%) of polyacrylamide (14). Gels were either stained with Coomassie brilliant blue or by the silver stain method of Oakley *et al.* (15).

RESULTS

Purification Procedure. The purification scheme used for the TTX binding component solubilized from eel electroplax membranes is summarized in Table 1. The introduction of an immunoaffinity purification step that used the monoclonal antibody secreted by the hybridoma clone VD10 (11), after an ion-exchange purification step, resulted in a 130-fold purification of the TTX binding component over the initial detergent extract, yielding a preparation that had a specific activity 1.5-fold greater than previously obtained.

Protein A-Sepharose chromatography. The immune complex formed by incubating the purified monoclonal antibody VD10 and preparation I was separated by affinity chromatography with a protein A-Sepharose conjugate. A large excess of antibody (11 μM) over TTX binding sites (0.43 μM) was used to facilitate rapid and complete immune complex formation. [^{3}H]TTX (1 μM) was added to this incubate to stabilize the TTX binding activity (4), and the [^{3}H]TTX binding activity was not lowered after immune complex formation.

The impure immune complex preparation was passed through a protein A-Sepharose column at pH 7.5 (Fig. 1). The TTX binding activity (33%) retained by the column at pH 7.5 was eluted at pH 6, and the NaDodSO$_4$/polyacrylamide gel pattern for these fractions indicated that a substantial purification had been achieved (Fig. 2, lane C), although a large excess of free IgG was coeluted with the immune complex. Later fractions eluted at pH 6 contained only antibody (Fig. 2, lane D).

DEAE-Sephadex chromatography. The excess IgG was separated from the purified immune complex by a further ion-exchange procedure. Fractions eluted at pH 6 from the protein A column and containing [^{3}H]TTX binding activity were pooled and passed through a small (1 ml) DEAE-Sephadex column (Fig. 3). The [^{3}H]TTX binding activity was recovered from the resin by increasing the ionic strength to 0.4 M NaCl. The overall recovery of both protein and binding activity was 50% (see Table 1). Inspection of the NaDodSO$_4$/polyacrylamide gel pattern of the fractions not retained by the resin indicated the exclusive presence of IgG (data not shown).

Because IgG was present in the most active fractions (Fig. 4, lanes C and F), it is likely that the immune complex was eluted from the column with 0.4 M NaCl and that it did not dissociate under these conditions. Indeed, it was difficult to dissociate the immune complex without destroying the highly labile [^{3}H]TTX binding activity.

The highest specific activity obtained for the immune com-

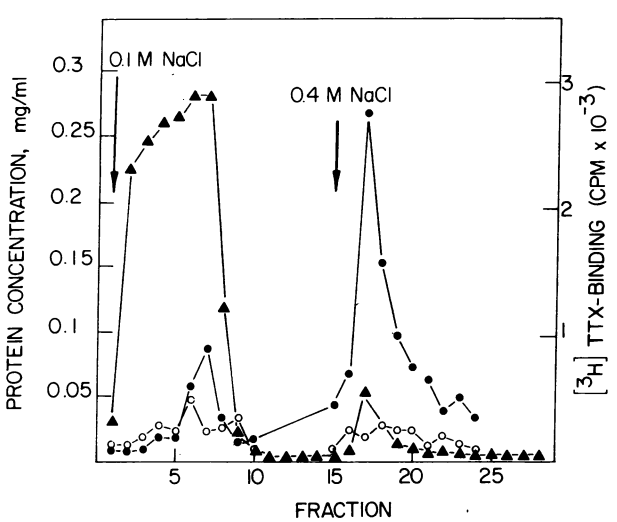

FIG. 3. DEAE-Sephadex A-25 chromatography of the TTX binding component—immune complex. ●, [^{3}H]TTX binding; ○, [^{3}H]TTX binding in the presence of 2 μM unlabeled TTX; ▲, protein concentration determined by the fluorescamine assay (13). Fractions 18–23 from the protein A column (Fig. 1) were pooled, 1 μM [^{3}H]TTX was added, and the sample was titrated gently to pH 7.5 with 1 M NaOH at 4°C; 0.1 M NaCl was added (final volume, 13.7 mls.). This material was passed through a 1-ml DEAE-Sephadex A-25 column, which had been preequilibrated with 0.1 M sodium phosphate containing 0.1% Lubrol/PtdCho, 0.05% NaN_3, protease inhibitors, 0.1 μM [^{3}H]TTX, and 0.1 M NaCl (pH 7.5), at 30 ml/hr (2-ml fractions were collected). After loading, the column was washed with 17.5 ml of this buffer. The [^{3}H]TTX binding activity was eluted with the same buffer containing 0.4 M NaCl (at this stage, 1-ml fractions were collected). The presence of 0.4 M NaCl did not lower the [^{3}H]TTX binding activity.

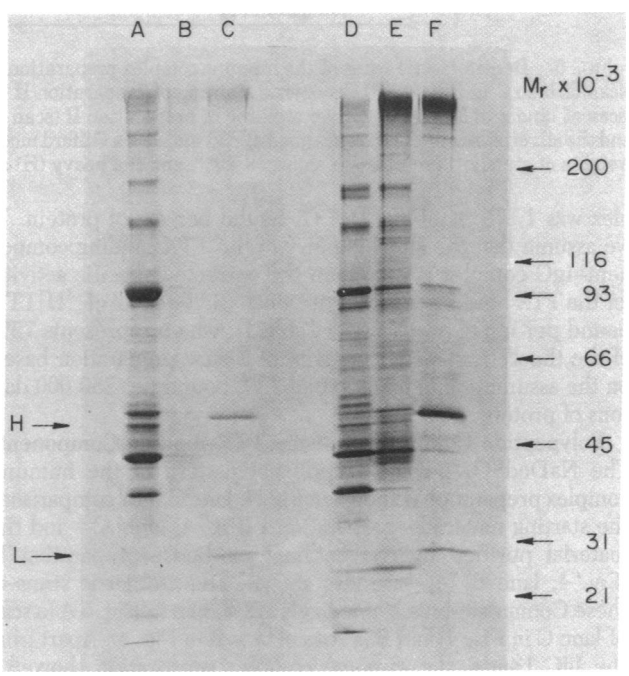

FIG. 4. Gradient NaDodSO$_4$/polyacrylamide gel (4–15%) electrophoresis under reducing conditions of preparation I, preparation II, and the immune complex preparation. Lanes: A and D, preparation I; B, preparation II—pooled and concentrated peak 3 fractions from a rechromatographed Sepharose 6B column (see ref. 4); C, pooled and concentrated peak 3 fractions from the 0.4 M NaCl elution of Fig. 3; E, preparation II—peak fraction from a rechromatographed Sepharose 6B column (specific activity, 1,870 pmol/mg of protein); F, peak fraction from the 0.4 M NaCl elution of Fig. 3 (specific activity, 1,811 pmol/mg of protein). The gel was stained with Coomassie brilliant blue (Lanes A, B, and C) or with silver (15) (Lanes D, E, and F). The molecular weights of standard proteins and the heavy (H) and light (L) chains of IgG are indicated.

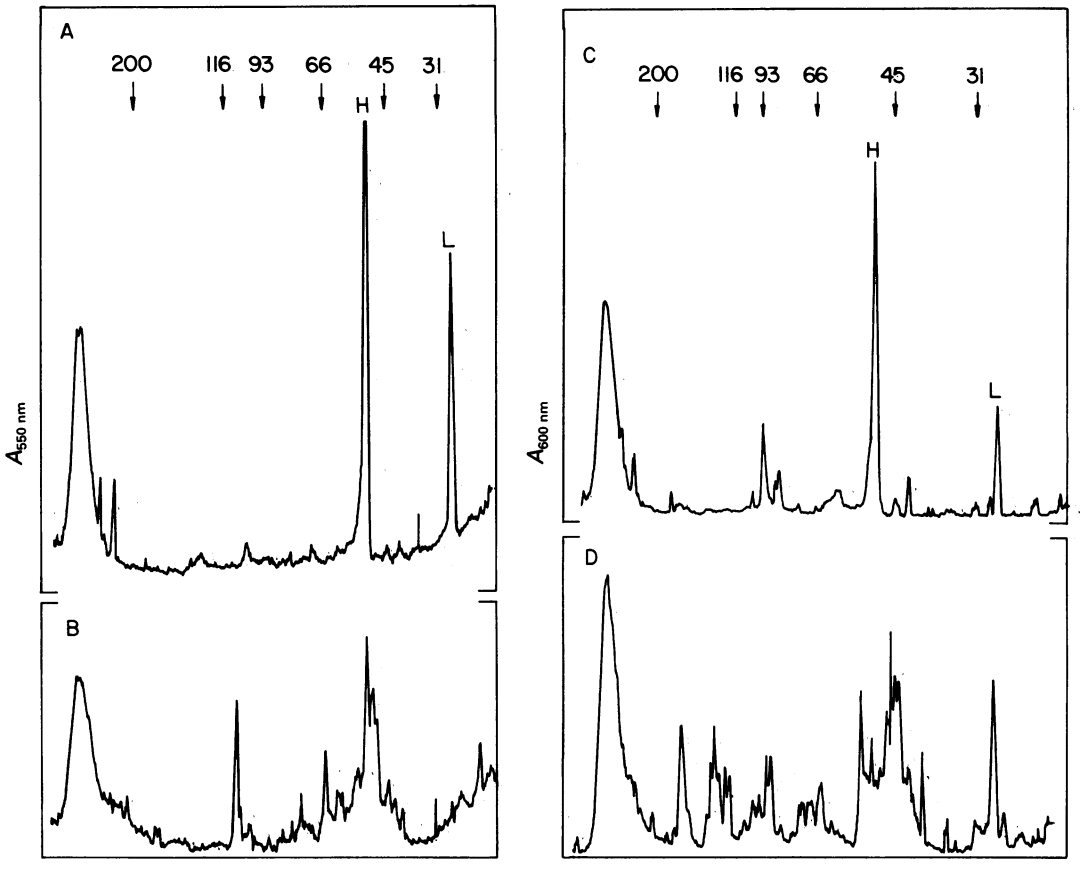

FIG. 5. Densitometric scans of the immune complex preparation and of preparation II. (*A*) Coomassie staining of immune complex preparation (scan of lane C in Fig. 4). (*B*) Coomassie staining of preparation II (scan of lane B in Fig. 4). (*C*) Silver staining of immune complex preparation (scan of lane F in Fig. 4). (*D*) Silver staining of preparation II (scan of lane E in Fig. 4). The Coomassie blue-stained gels were scanned at 555 nm, and the silver-stained gels were scanned at 600 nm with a Gilford model 240 spectrophotometer equipped with a linear transport unit. The molecular weights of standard proteins are shown $\times 10^{-3}$, and the heavy (H) and light (L) chains of IgG are indicated.

plex was 1,875 pmol of [^{3}H]TTX bound per mg of protein. If we assume that the stoichiometry of the TTX binding component–IgG complex is 1:1, then the corrected specific activity for the TTX binding component alone is 3,000 pmol of [^{3}H]TTX bound per mg of protein (see Table 1), which represents 75% of the theoretical specific activity of a pure preparation based on the assumption of one mole of TTX bound per 250,000 daltons of protein.

Polypeptide Composition of the TTX-Binding Component. The $NaDodSO_4$/polyacrylamide gel pattern of the immune complex preparation is shown in Fig. 4, lane C. For comparison, the starting material—preparation I (Fig. 4, lane A)—and the material purified by conventional methods—preparation II (Fig. 4, lane B) (4)—are also shown. Densitometric scans of these Coomassie-blue stained gels are shown in Fig. 5 *A* (a scan of lane C in Fig. 4) and *B* (a scan of lane B in Fig. 4). Apart from the IgG bands, the immune complex preparation showed a much simpler gel pattern than did preparation II (4); the major polypeptide present was of M_r 250,000.

$NaDodSO_4$/polyacrylamide gels were also subjected to the more sensitive silver staining method (15). The gel patterns (Fig. 4, lanes D, E, and F; Fig. 5 *C* and *D*) show the purity of the material obtained (Fig. 4, lane F). It is worthwhile noting that the specific activity of preparation II (Fig. 4, lane E; 1,870 pmol/mg of protein) was approximately the same as the uncorrected specific activity of the immune complex preparation (Fig. 4, lane F; 1,811 pmol/mg of protein). In addition, the specific activities obtained for preparation II in the present report are comparable to those reported previously (4, 5).

The results of five immune complex preparations gave a gel pattern essentially similar to those in Fig. 4, lane F and Fig. 5*C*. Apart from the antibody bands, the major protein species present was a diffuse band of M_r 250,000 that was associated with sometimes one, sometimes two sharp bands, less intensely stained. In addition, minor bands with M_rs of ≈95,000 (doublet), ≈44,000, and ≈23,000 were always observed. Occasionally, a varying number of bands of M_rs 40,000 and 50,000 were observed, but these were minor contaminants of the IgG (data not shown).

DISCUSSION

This report demonstrates the use of the antibody secreted by the monoclonal hybridoma line VD10 (11) to purify the TTX binding component of the voltage-sensitive Na^+ channel from *E. electricus* electroplax. The immune complex was purified from a very heterogeneous preparation by the use of protein A-Sepharose chromatography. Protein A binds to the Fc fragment region of the antibody molecule (16), so the use of this type of immunoaffinity resin circumvents the loss of antigenic activity that occurs when antibody is coupled to a support matrix. This technique also has another advantage: the elution conditions of the immune complex are very mild, thus avoiding the destruction of TTX binding activity by chaotropic agents or low pH.

Only one third of the [^{3}H]TTX binding activity applied to the protein A column was recovered at pH 6. A large excess of pure VD10 IgG was used; therefore, it is unlikely that complete immune complex formation had not occurred. A possible explanation for the low recovery of immune complex is that it is of

sufficient size to be partially excluded from the Sepharose support matrix whose gel exclusion properties are likely to have been changed due to the high degree of substitution by protein A.

Examination of the $NaDodSO_4$/polyacrylamide gel pattern demonstrated that the immune complex preparation was highly purified, as suggested by its high specific activity. The large molecular weight species (M_r, ≈250,000), previously shown to be a component of the eel Na^+ channel (4, 5) predominated. Peptides with M_rs of ≈95,000 (doublet), ≈44,000, and ≈23,000 were also present, although in low amount. Interestingly the components with M_rs of ≈95,000 and ≈44,000 are present in preparations I and II in much larger quantities, suggesting that these may be contaminants.

A large molecular weight species (M_r, 270,000) was shown to be a component of the rat brain Na^+ channel (7, 10). In addition, a low molecular weight species (M_r, 33,000–38,000) was observed in those studies. Recently, Weigele and Barchi (9) have claimed that a large glycoprotein is present in a rat muscle sarcolemma Na^+ channel preparation, in addition to lower molecular weight components. To what extent the low molecular weight components observed in these two studies correlate with the low molecular weight peptides identified in the present report is unclear. It would appear, however, that the major structural species of the Na^+ channel TTX binding component is a high molecular weight glycopeptide (M_r, ≈250,000). The purification procedure outlined in the present report offers the best available method for obtaining this high molecular weight polypeptide from eel electroplax of purity sufficient for structural studies.

We thank Drs. Jeremy P. Brockes, Larry Fritz, and Katie Stygall for helpful discussions concerning many aspects of this work and for the gifts of immunoreagents. In addition, we thank Dr. Larry Fritz for growing VD10 in culture and helping with ascites fluid production. Laurie Jutzi typed the manuscript, and Valerie Purvis produced the artwork. This research was supported by U.S. Public Health Service Grants NS 12018 and GM 16424 and by a grant from the Pew Charitable Trust.

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